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The Sorting of Cells

Although early flow cytometers were developed as instruments that could separate or sort particles based on analysis of the signals coming from those particles, it is now true that most flow cytometry involves analyzing particles but not actually sorting them. This is just one of many examples of the way in which flow technology has moved in unpredictable directions. Many cytometers now do not even possess a sorting capability, and the instruments that can sort particles may be used only infrequently for that purpose. Nevertheless, sorting cells or chromosomes with a flow cytometer is an elegant technology; it may be the only method available for obtaining pure preparations of particles with certain kinds of characteristics of interest. On both of those counts it is certainly worth knowing about sorting even if you are not interested in using the technique at the moment. Now that you are comfortable with flow cytometry and its basic applications, this chapter will present a bit more technical information about the way a sorting flow cytometer (Fig. 9.1) can be used to sort cells.

SORTING THEORY

If a stream of liquid is vibrated along its axis, the stream will break up into drops (pick a nice summer day and try it with a garden hose). The characteristics of this drop formation are governed by an equation that will be familiar to anyone who has studied light waves: $v = f\lambda$, where v is the velocity of the stream, f is the frequency of the vibration applied, and λ is the “wave length” or distance between the drops. One other fact that needs to be known before we can under-



Fig. 9.1. A sorting flow cytometer (MoFlo by Cytomation). The outward complexity of this instrument compared with benchtop cytometers (see Fig. 1.5) reflects the electronic controls necessary for sorting as well as the adaptability of research cytometers with regard to multiple lasers and to the filters and mirrors in the optical light path for multiparameter analysis.

stand sorting is that drops form in a regular pattern so that the distance between drops is, under all conditions, equal to just about 4.5 times the diameter of the stream. For the usual flow cytometer, if a nozzle with a 70 μm orifice is used, the drops will form with a wavelength of about 315 μm . In most cytometers, the stream velocity is set at approximately 10 m/s. Because λ (315 μm) and v (10 m/s) are both now determined, the frequency to achieve drop formation is fixed as well. The nozzle needs to be vibrated at

$$f = (10)/(315 \times 10^{-6}) = 31,746 \text{ cycles per second}$$

to get drops to form (and this kind of restrictive relationship between v , f , and λ is why drops will form from the garden hose only when you get it vibrating at a certain frequency). Figure 9.2 illustrates this relationship between drop generation frequency, stream velocity, and

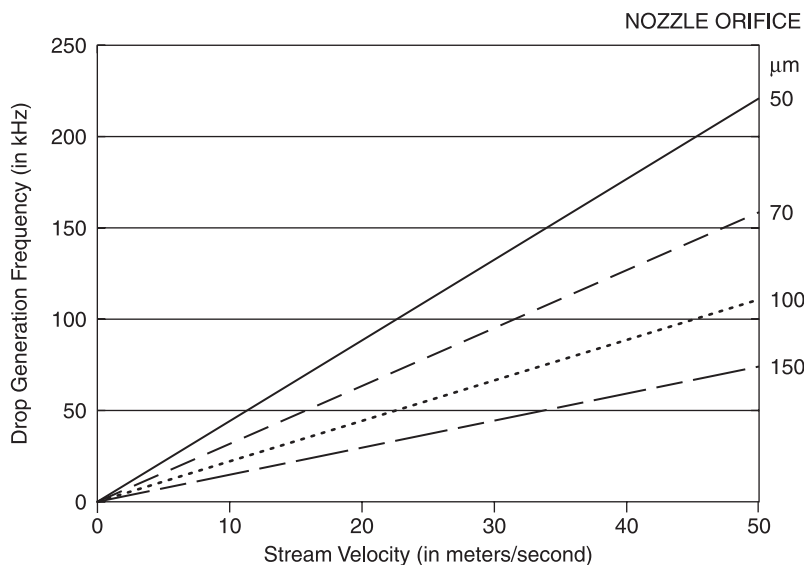


Fig. 9.2. The vibration frequency required to generate drops is determined by the stream velocity and the nozzle orifice diameter. Faster drop generation permits faster cell flow rate without enclosing significant numbers of multiple cells in single drops.

nozzle orifice size. With a flow nozzle vibrating at about 30,000 cycles per second, if the sample is of a concentration such that particles are moving down that stream at 30,000 particles per second, there will be, on average, one particle in every drop; if the particles are less concentrated and flowing at 3000 particles per second, there will be, on average, one particle in every tenth drop.

When a stream vibrates, drops break off from the jet at some measurable distance from the fixed point of vibration. Therefore a particle can be illuminated and its signals detected without interference as it flows within the core of a stream through a vibrating nozzle—as long as the illumination and detection occur close to the point of vibration (Fig. 9.3). At some distance after the point of analysis, the stream will begin to break up into drops; particles will be contained in some of those drops as they break off. It remains now for the cytometer to put a charge on the drops containing particles of interest. Because the drops will flow past positively and negatively charged high-voltage plates, any drop carrying a positive or negative

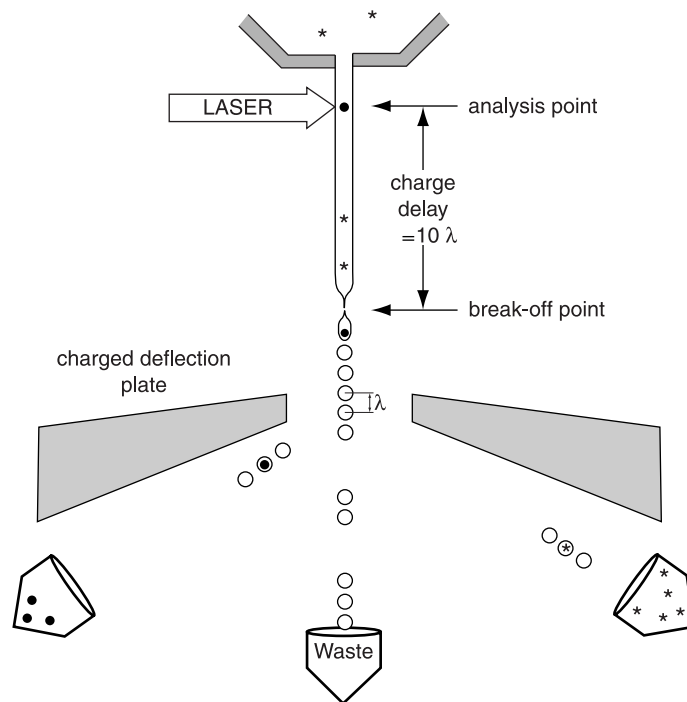


Fig. 9.3. Droplet formation for sorting. A time delay is required between analysis of a particle and the charging of the stream so that only the drop (or three drops) surrounding the desired particle will be charged and then deflected.

charge will be deflected out of the main stream and toward one or the other plate. Once this happens, it is easy to place test tubes, Eppendorf tubes, or wells of a microtiter plate in position to catch the drops deflected to the left or right.

The main trick of flow sorting is, therefore, to apply a charge only to the correct drops (i.e., the ones containing the desired particles) and to no others. To do this, we need to know the time that elapses between the moment a particle is analyzed at the analysis point and the moment, a bit later, that that particle is just about to be trapped in a newly formed drop separating from the stream. If the entire stream is charged (either negatively or positively) by applying a charge at the nozzle just before the drop is formed with the desired particle within it, and then the entire stream is grounded at the nozzle to remove that charge just after the drop in question is formed and

has detached from the stream, then only the drop with the desired particle within it will remain charged. Therefore, all we need to do is determine the time it takes a particle to move from the analysis point to the point where the drop containing it breaks away from the stream.

Different methods are available for determining the time delay between illumination of (and detecting signals from) the particle and the trapping of that particle in an isolated drop. In some systems, the operator does a “test sort matrix” of sorted cells or fluorescent beads into puddles on a slide. By changing the timing of the drop charge, the operator can check (with a microscope) to see which time delay produces sorted cells or beads in the puddle. In other systems, we can measure the distance between the analysis point (the laser spot on the stream) and the drop break off point. Then, to convert that distance into a time, we count the number of drops that occur in an equal distance measured further down the stream. Because we can know the frequency of vibration that is driving the nozzle (in cycles per second), we therefore also know the number of seconds per cycle. By counting the number of drops that occur in a distance equal to the distance between analysis point and drop break-off point, we can convert the counted number of drops into a time in seconds. It is that time that is the time delay we want between illumination of the particle and charging the stream. Fortunately, most cytometers will do most of these calculations for us. We simply need to count the number of drops that occur in a distance equivalent to the distance between analysis point and drop break-off point. The cytometer electronics then converts that drop number into a time delay, being the amount of time we need to delay our charging of the stream so that a particle analyzed at the laser intercept and found to have desired characteristics has moved in the stream and is about to be trapped in a forming drop.

Once the charge delay time has been determined (either by counting drops or by a test matrix), the cytometer can then be given sorting regions delineating the flow cytometric characteristics of the desired particles. These regions are, like any flow cytometric regions, simply the numbers (on a scale of 0–1023) that describe the intensity of the light signals characterizing the particles of interest. They can involve single flow parameters (e.g., a particular range of green fluorescence intensity), or they can involve multiple parameters and combinations of regions (e.g., a forward and side scatter region combined with a

range of green, orange, and red intensities defining the phenotype of a subset of cells). If we are at all unsure about our accuracy in timing the delay for the drop charge, we can charge the stream for a slightly longer time (centered around the calculated time delay) so that two or three drops are charged after a desired particle has been detected at the analysis point. This should ensure that our desired particle is deflected, even if it should move slightly faster or slightly more slowly than anticipated.

As discussed earlier, with a nozzle of 70 μm and a stream moving at 10 m/s, our system is committed to a vibration frequency of about 30,000 cycles per second (30 kHz) in order to get drops to form. If we prefer a margin of safety, we may want to charge and sort three drops at a time; in that case, we will want a particle in no more than every third drop. This means that our total particle flow rate can be no faster than 10,000 particles per second. Because cells are not spaced absolutely evenly in the flow stream (they obey a Poisson distribution), most sorting operators like to have particles separated by about 10–15 empty drops. With a 70 μm nozzle and a stream velocity of 10 m/s, this restricts our total particle flow rate to about 2000–3000 particles per second. For sorting cells of very low frequency within a mixed population, this may involve unacceptably long sorting times.

The startling implications of this restriction on particle flow rate according to the frequency of drop generation can be seen in Table 9.1. If, in order to separate cells by 15 empty drops, the rate of particles flowing is restricted to 2000 per second, then the particles we

TABLE 9.1. Cell Sorting: Time Needed to Sort Required Number of Desired Particles at Total Particle Flow Rate of 2000 Particles per Second

Required No. of desired particles	Desired particles as % of total particles			
	0.1%	1.0%	5.0%	50.0%
10^3	8.3 min	50 s	10 s	1 s
10^4	1.4 h	8.3 min	1.7 min	10 s
10^5	14 h	1.4 h	17 min	1.7 min
10^6	5.8 d	14 h	2.8 h	17 min
10^7	1.9 mo	5.8 d	1.2 d	2.8 h
10^8	1.6 yr	1.9 mo	12 d	1.2 d

actually desire to purify by the sorting procedure will be flowing at something less than that rate. If the desired particles are 50% of the total, then they will be flowing at a rate of 1000 per second (3.6 million per hour). If, however, the desired particles are only 0.1% of the total, they will be flowing at a rate of 7200 per hour. The time taken for sorting cells, then, depends first on how fast your cells can be pushed through the cytometer without unacceptably high rates of coincidence with multiple cells in drops; second, on what percent the desired particles are of the total number of particles present; and third, on how many desired particles are actually required at the end of the day (or month!). Scientists tend to think logarithmically: It is easy to say that perhaps 10^6 or 10^7 sorted particles are required for a given application. But sorting 10^7 particles takes 10 times longer than sorting 10^6 particles; this can represent a very large investment in time. One moral of this story is that any preliminary procedure that can be applied to enrich a cell suspension before flow sorting will save considerable amounts of time.

Of course, with a little thought it should also be clear that cells could be run through the sorter more quickly without the risk of multiple cells in drops if the drops could be generated at a faster rate. According to our equation, this can be done either by making the nozzle orifice diameter smaller or by running the stream at a higher velocity. The first solution tends to be impractical, as a clogged nozzle ends up being very slow in the long run. The second solution was implemented initially at the Livermore and Los Alamos laboratories, where a high-pressure system (about 100–200 psi) brings about a stream velocity of about 40–50 m/s and a droplet frequency of 150,000–200,000 drops per second. As a result, particles flowing at rates of 10,000–20,000 particles per second can be sorted without significant risk of multiple cells in a drop. Total sorting times can be cut by a factor of 10. High-speed sorting has recently become available on commercial instruments. Modifications of tubing strength, electronics dead time, and air controls have permitted the pressure on the sheath tank to be increased from about 10–15 psi to 30–100 psi. This pressure brings about an increase of the stream velocity from 10 m/s to about 20–40 m/s. Drop drive frequencies need then to be increased (according to the equation) to 63 to 127 kHz (with a 70 μm nozzle) to produce drops. Cells can therefore flow at 6000 to 13,000 cells per second and still appear (on average) in only every tenth

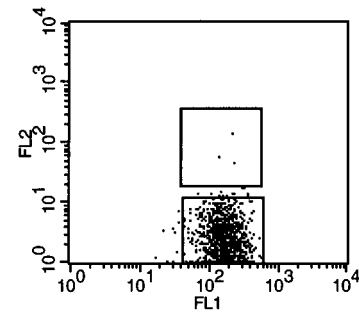
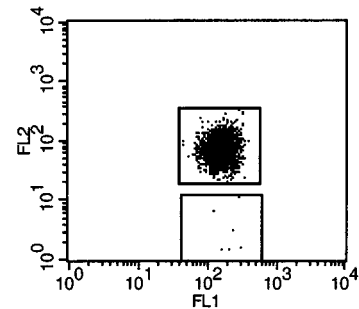
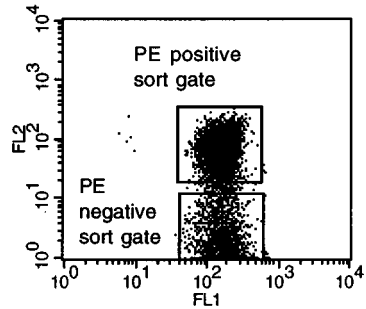
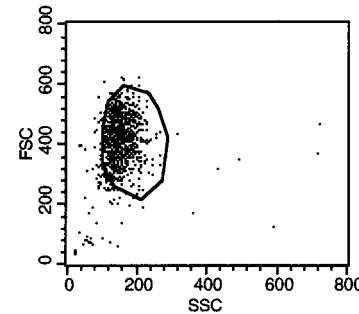
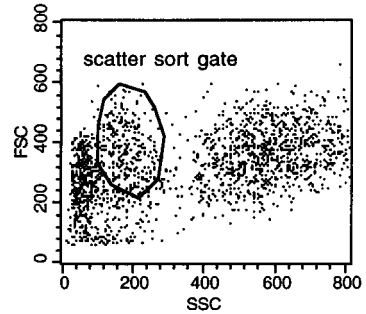
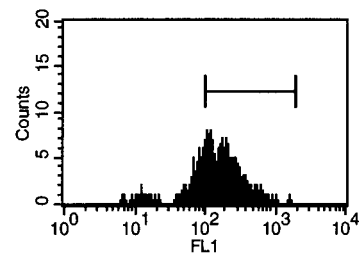
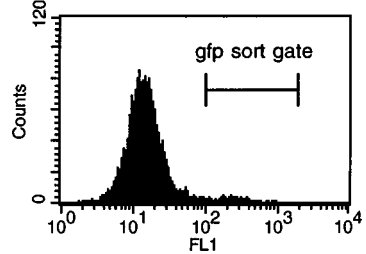
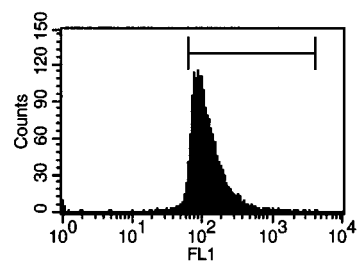
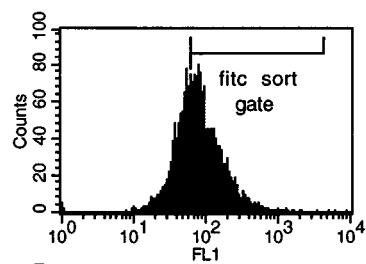
drop. If higher abort rates can be tolerated, then these flow rates can be tripled (with one cell in, on average, every third drop).

CHARACTERIZATION OF SORTED CELLS

The first matter of concern after a sort is always purity, but purity is never a straightforward issue (just like many other aspects of flow cytometry). Sorting of test beads (as a bench mark) routinely gives purity of better than 99%. Figure 9.4 shows four real-world examples of cells before and after sorting. In Figure 9.4, the sort regions on the plots in the right column (the sorted cells) have not been moved from where they were set as sort gates before the sort (left column). These plots illustrate that, at times, the accuracy of the sorter may appear to be suspect. Our good results with beads should, however, lead us to conclude that the problem with cells may have nothing to do with the accuracy of the flow cytometer.

The sort may appear to be inaccurate (and the sorted cells less than perfectly pure) because the actual intensity of the cells may decrease between the time a cell is sorted and the time it is run back through

Fig. 9.4. Four examples of cells sorted by Gary Ward in the Englert Cell Analysis Laboratory. The plots in the column on the left are from cells before the sort, indicating the region used to set the sort gate; the column on the right shows the sorted cells, run back through the cytometer for a “purity check.” The top row shows mouse B lymphocytes that have been transfected with a gene for the human IgA Fc receptor (a mixed clone cell line from M van Egmond and J van de Winkel; sort experiment by Mark Lang); the cells have been stained for the IgA Fc receptor and then sorted for the top 50% expressors. The second row down shows data from a cell line derived from the tracheal epithelium of a cystic fibrosis patient and invaded by *Staphylococcus aureus* bacteria carrying a plasmid promoter library driving the expression of green fluorescent protein (GFP); GFP expression within the eukaryotic cell (but not in vitro) allows the sorting of cells containing bacteria with promoters activated by intracellular signals (data courtesy of Niles Donegan). The next row down shows monocytes sorted on the basis of their forward and side scatter signals from a preparation of white cells from human peripheral blood (data from Gary Ward). The bottom two rows illustrate data (from James Gorham) for mouse spleen cells (pre-purified with anti-CD4 magnetic beads) stained with anti-CD4-FITC and anti-CD62L-PE (a marker for naïve cells); the two-way sort separated naïve and memory CD4-T cells in order to compare their biological activities in vitro.



the instrument for the purity check. Additionally, the same cell may show slightly different fluorescence each time it is run through the cytometer due to fluctuations in the optics or fluidics of the system. Another reason for “impurities” results from the possibility of aggregates in the original suspension that dissociate after sorting. An aggregate of a positive and negative cell will be sorted as if it is a positive cell and then may disaggregate into one of each. For these reasons, sorting is often a matter of judgment as well as skill.

Aggregates can be avoided in the sort to some extent by using very tight forward/side scatter regions. For reasons of changing fluorescence intensity during a long sort, however, the purity of a sort may often need to be judged by standards that are different from those used to drive the sort decisions of the cytometer. Readers should look at the sorted cells in the right column of Figure 9.4 and guess at what factors may have contributed to less than perfect purity. In any case, a sort operator will ascertain the characteristics of the cells that are desired, set a sort gate that may be considerably tighter than those desired characteristics, and then check the success of the sort by comparing the sorted cells with those that were desired. Depending on the sophistication of the scientist commissioning the sort, all of this may be explicit or may just be part of the secret communication between the operator and his or her instrument.

At the end of the day, a flow sort needs to be characterized not only by the *purity* of the sorted cells according to the desired criteria but also by the *efficiency* of the sorting of the selected cells from the original mixed suspension (with low loss) and by the *time* taken to obtain the required number of sorted cells. In most cytometers, purity is good (understanding that apparent failures in purity may have legitimate reasons, as described above). Purity is well protected by electronic fail-safe controls that abort drop charging when cells are so close together at the analysis point that they may be enclosed in the same drop downstream (or in adjacent drops if two or three drops are sorted together). In this way, high cell flow rates will lead to lost cells and will therefore compromise the sorting efficiency but may not compromise purity until cells are so close together that they are coincident in the laser beam. As cell flow rates are increased by using more concentrated suspensions, more sort decisions may be aborted (leading to poorer and poorer efficiency), but the speed of sorting of the desired cells will increase until the cell flow rate gets so high and aborted sorts become so frequent that the actual speed of sorting of

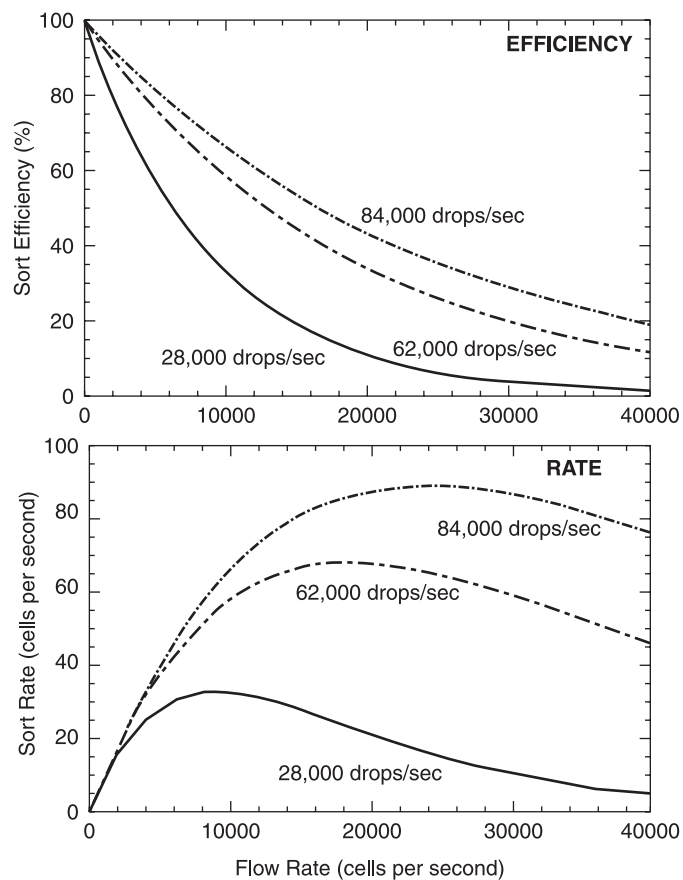


Fig. 9.5. Efficiency and speed of sorting are affected by the flow rate of cells. At high flow rates, more desired cells are lost, but the speed of collecting these desired cells increases until the loss of efficiency becomes greater than the increase in speed. High-speed sorting, with more drops per second, increases the efficiency and decreases the time required to obtain the desired number of cells. The model from which these graphs were generated was derived by Robert Hoffman: for these data, a three-drop sort envelope was used, 1% of the cells were “sorted,” and the electronic dead time was set at 6 μ s. If one drop is sorted with each sort decision (instead of three), the theoretical efficiency of the sorting improves considerably (as does the rate of collecting the sorted cells).

the desired cells starts to drop off (Fig. 9.5). This figure also indicates how beneficial high drop generation frequencies (high-speed sorting) can be for both the rate of sorted cell collection and also the efficiency of the sort.

Whether at high or low speed, every sort needs to be optimized according to the particular requirements of the experiment in question. Speed, purity, and sorting efficiency interact and are not, all three, optimizable under the same conditions. For example, a sort for a minor subpopulation of cells from a large, easily obtainable cell suspension may require optimization of speed, without concern about low sorting efficiency. On the other hand, a requirement for as many specific cells as possible from among a scarce mixed suspension will need to maximize recovery without much concern for speed.

ALTERNATIVE METHODS FOR SORTING

There are, of course, other methods for separating cells. Traditional methods have been based on physical properties (e.g., density gradient centrifugation) or on biochemical properties (e.g., the adherence of T cells to sheep erythrocytes or of monocytes to plastic or the resistance of white cells [as opposed to erythrocytes] to lysis by ammonium chloride). Additional separation methods involve the use of complement-fixing antibodies, which can be used to stain unwanted cells; complement lysis will enrich the suspension for the unstained cells. More recent methods involve the staining of cells with antibodies that have been bound with magnetic beads; the cells of choice, after being stained with the antibodies, become magnetic and can be removed from a mixed suspension by passing the suspension through a magnetic field.

All these methods are “bulk” or “batch” methods; that is, the amount of time that it takes to perform a separation is not related to the number of cells that are being separated. For example, if blood needs to be centrifuged for 20 minutes over a density gradient in order to separate monocytes and lymphocytes from neutrophils and erythrocytes, the separation will take 20 minutes whether the original volume of blood is 1 or 100 ml (assuming enough buckets in the centrifuge). Batch procedures are therefore ideally combined with flow sorting; they provide an initial rapid pre-separation that can greatly decrease the amount of time required for the final flow separation (refer back to Table 9.1).

By way of a practical example, consider CD5-positive B lymphocytes. Because CD5-positive B lymphocytes may be 10% of all B

lymphocytes, B lymphocytes are perhaps 10% of all lymphocytes, and lymphocytes could be 50% of all mononuclear white cells, if we want to sort out CD5-positive B cells (0.5%) from a mononuclear cell preparation, they will be flowing through our system at a rate of 36,000 per hour if our total particle flow rate is limited to about 2000 per second. It would therefore take 28 h to collect 1 million CD5-positive B cells. However, if we remove all nonlymphocytes by adherence to plastic and then remove all T lymphocytes by rosetting with sheep erythrocytes (both techniques are rapid batch processes that might take an immunologist about an hour to perform), we can then send pure B lymphocytes through the cytometer. The desired CD5-positive particles (now 10% of the total) will therefore be flowing at a rate of 720,000 per hour. As a result, the time it will take to get 1 million desired cells will go from 28 hours to 1.4 hours. This has obvious benefits both in terms of cost, if you are paying for cytometer time, and the general health and viability of the sorted cells at the end of the procedure.

Batch procedures can be useful as pre-flow enrichment of the cells of interest. They can also be used as alternatives to flow sorting if the cells of interest can be appropriately selected. Flow sorting, although slow, is generally the only alternative if multiple parameters are required to define the cells of interest, if antigen density on the cell surface is low, or if forward or side scattered light is part of the definition of the desired cells.

In recent years, alternative flow cytometric methods for sorting have been developed. Instead of sorting cells by applying a charge to the drops generated by a vibrating stream, one of these methods involves sorting cells according to their flow signals by “grabbing” the cells of interest into a catcher tube that moves into the center of the stream when a desired cell flows by. Another flow sorting technology involves the use of a piston that applies pressure to one arm of a Y-shaped channel downstream from the laser intercept. Cells normally flow down the waste arm of the “Y.” By application of pressure after a desired cell flows through the analysis point, selected cells are diverted from the waste arm to the sorting arm of the “Y.” While these are cheaper alternatives than traditional, droplet-based, electronic sorting, speed limitations and the large volume of sheath fluid that dilutes the sorted cells make these methods primarily suitable for collection of small numbers of cells.

Another method for flow sorting is currently under development. It permits extremely rapid selection of cells at speeds beyond those possible even under very high-speed traditional (drop deflection) conditions. In drop deflection sorting, the sort rate is restricted by the rate of drop formation. This alternative method could be called *reverse sorting* or *optical zapping* and does not involve drop formation. Cells or chromosomes are stained with a phototoxic dye as well as with antibodies or a DNA stain. The particles are sent through a more or less conventional cytometer. Downstream from the analysis point, a cell or chromosome that does *not* possess the light scatter or fluorescent properties of interest is zapped by a second (killer) laser that activates the phototoxic dye. All particles end up in the collection vessel, but only those that have not been zapped survive. The rate of selection/destruction is limited, therefore, only by the rate at which the zapping laser can be deflected away from the stream. In proof-of-principle experiments, it appears that sorting rates may be increased 5-fold over those of the high-speed sorters and 25-fold over conventional sorters.

THE CONDITION OF CELLS AFTER SORTING

This brings us to a brief discussion of the condition of cells after they have been sorted. Depending on the purpose for which the sorted cells will be used, there may be different requirements for sterility and viability. If you are sorting cells for subsequent polymerase chain reaction amplification, for example, then neither sterility nor viability is important. On the other hand, if you are using a flow sorter because you want to clone cells expressing a certain characteristic, then both sterility and viability are necessary. Sterile sorting is possible (with care and a fair amount of 70% ethanol used to sterilize the flow lines). Sorted cells can remain sterile through the sort procedure and can subsequently be cultured for functional analysis or cloning. Given that cells in a sorting cytometer have been subjected to acceleration, decompression, illumination, vibration, enclosure, charging, and deflection toward high voltage plates, it seems reasonable to expect some trauma and, therefore, to coddle the cells as quickly as possible after the sort if viability is required. If you remember that the cells move down the stream in a core that is just a small percentage of the total volume of the sheath stream, you will realize that each drop is

mainly sheath fluid and that, following drop deflection, the cells are deposited in medium that is partly sample medium, but mainly sheath fluid. Therefore, it is recommended to sort the cells into tubes that already contain a volume of appropriate culture medium. Some cells lose viability after sorting; many cells seem to be remarkably oblivious to the process.

It is also important to remember that most sorting procedures involve staining of the cells with antibodies conjugated to fluorochromes so that the cells can be sorted according to whether or not they are fluorescent. There is always the possibility that the binding of antibodies to the cell surface might by itself affect the function of the selected cells. In other words, the sorted cells that are low in fluorescence intensity may function differently from the cells that are bright not because they are essentially different but simply because the staining itself has blocked or tweaked surface receptors. By now you should be able to design a control for this potentially confusing phenomenon. If the staining is functionally neutral, stained cells (without any sorting at all) should function identically to unstained cells with regard to the assay of interest. In addition, it is important to be sure that the sorting procedure does not affect cell function. The control for this is to compare unsorted cells with cells that have gone through the sorter with large sort gates that do not actually exclude any cells. The sorting procedure should not change the functional ability of the cells if the proportion of different kinds of cells is not changed in this control sort. If the functional ability of the cells is changed either by sorting or by staining, then this needs to be considered in the interpretation of the subsequent functional analysis of the sorted populations.

FURTHER READING

Practical issues in sorting are discussed well in Chapter 4 of **Ormerod** and in Section 7 of **Diamond and DeMaggio**.

Theoretical principles of droplet generation sorting are discussed in Chapter 8 in **Melamed**, in Chapter 6 of **Watson**, and in Chapter 3 of **Van Dilla**.

High speed sorting is described in Section 1.7 of **Current Protocols in Cytometry**.

For discussion of chromosome sorting by optical zapping, see Roslaniec MC, Reynolds RJ, Martin JC, et al. (1996). Advances in flow cytogenetics: Progress in the development of a high speed optical chromosome sorter based on photochemical adduct formation between psoralens and chromosomal DNA. NATO Advanced Studies Series, Flow and Image Cytometry 95:104–114.